IN THE UNITED STATES PATENT & TRADEMARK OFFICE

In re Application of:	) For:	Enhanced Herbicide Composition
	)	
Silverman, et al.	)	
	)	
Serial No.: 10/619,347	) Group Art Unit:	1616
	)	
Filed: July 14, 2003	) Examiner:	S. Clardy

DECLARATION UNDER 37 C.F.R. §1.132

Mail Stop Patent Applications
Commissioner for Patents
P. O. Box 1450
Alexandria, Virginia 22313

Sir:

1. I, F. Paul Silverman, am an inventor of this application.

2. I received my Doctorate in Plant Biology from Rutgers University, New Brunswick, New Jersey USA in December 1994.

3. I have been employed by Valent BioSciences Corporation, the assignee of this application, since January 2000.

4. I currently hold the position of Senior Scientist.

5. I have read and understand this application including the claims, the Office Action dated March 30, 2006 and the cited and applied prior art.

6. The experiments performed by me or under my supervision show that salicylate potentiation of herbicidal activity of PSII does not work through NO_x, and therefore, the Office Action's assertion that "Klepper clearly suggests that the application of SA will synergistically enhance the activity of PSII herbicides" is incorrect.

Klepper (1988, Pest. Biochem. Physiol. **32**:173) investigated the effects of salicylic acid on nitrate and nitrite metabolism, and tested the hypothesis that the combination of PI (PSII

inhibitors) herbicides and salicylic acid could increase the production of NO_x by plant leaves *in vitro*.

Nitrogen oxide gases (NO_x) is a term for a group of reactive gases, all of which contain nitrogen and oxygen in varying amounts. Nitric oxide (NO) is the principal component in NO_x (See Klepper, abstract). Most of the NO_x gases are anthropogenic in origin and are common atmospheric pollutants. In plants, NO is the primary gas produced (see Klepper, p 174, second line). Over the last decade, NO has been identified as physiologically important to plants. Klessig et al. (2000, PNAS USA **97**:8849), highlighted the role of NO in the plant defense response. The recent cloning of the NO synthase (NOS) underscored the role of NO in ABA-regulation of stomatal closure (Guo et al. 2003, Science **303**:100). Moreover, NO has recently been shown to be involved in the regulation of time of flowering (He et al., 2004. Science **305**:1968). Thus, NO has been shown to be a signal molecule in regulation of several aspects of plant development.

Klepper demonstrated that salicylate treatment increased the production of NO_x in darkness. In contrast, neither salicylate nor PI herbicides alone produced NO_x in light. Only the application of both salicylate and PI herbicides together caused NO_x accumulation in light. According to Klepper's data, NO_x does not accumulate when plants treated with PI herbicides are incubated in light (See Table I, p. 177). This finding strongly suggests that NO_x accumulation is unrelated to herbicidal activity of PI herbicides, since PI herbicides act only in a light environment. Our data demonstrate that light is needed for atrazine (a typical PI herbicide) activity. (See Table 7 in the Application). The Herbicide Handbook (Weed Science Society of America, 1994, p.21) describes the mode of action of atrazine as follows:

> **"Mechanism of action**: Inhibits photosynthesis by binding to the Q_B binding niche on the D1 protein of the photosystem II complex in chloroplast thylakoid membranes, thus blocking electron transport form Q_A to Q_B. This stops CO_2 fixation and production of ATP and $NADPH_2$ (all needed for plant growth), but plant death occurs in most cases by other processes. Inability to reoxidize Q_A promotes the formation of triplet state chlorophyll that interacts with ground state oxygen to form singlet oxygen. Both triplet chlorophyll and singlet oxygen can abstract a hydrogen from unsaturated lipids and initiate a chain reaction of lipid peroxidation. Lipids and proteins are attacked and oxidized, resulting in loss of chlorophyll and carotenoids and in leaky membranes which allows cells and cell organelles to dry and disintegrate rapidly."

From this paragraph, it is clear that the activity of PI herbicides is directly related to light. Therefore, herbicidal damage caused by PI herbicides is not caused by the production of NO_x.

Our data conclusively prove that salicylate potentiation of atrazine is independent from whatever effects NO may have on plants. To demonstrate this, we utilized NO-releasing chemicals. One of these, sodium nitroprusside (SNP), readily decomposes to yield NO. We used SNP to test whether salicylate (SA) potentiation of atrazine is NO dependent. The results are shown in Table 1.

Table 1. Effect of the NO generator sodium nitroprusside (SNP) on sodium salicylate (NaSA) potentiation of atrazine herbicidal activity on tobacco.

Line No.	Treatments	48h post spraying	72h post spraying
		Percent leaf area Damaged	
1	Crop Oil Concentrate, 0.25% (v/v)	0 A	0 A
2	NaSA, 800 mg/l + COC 0.25%	1.1 A	1.1 A
3	SNP, 1490 mg/l (5 mM) + COC 0.25%	2.0 A	2.5 A
4	NaSA, 800 mg/l + SNP, 1490 mg/l + COC 0.25%	3.5 A	4.5 A
5	Atrazine, 250 mg/l + COC 0.25%	51.5 B	79.8 B
6	Atrazine, 250 mg/l + NaSA, 800 mg/l + COC 0.25%	73.5 C	93.8 D
7	Atrazine, 250 mg/l + SNP, 1490 mg/ l + COC 0.25%	62.5 BC	82.8 BC
8	Atrazine, 250 mg/l + NaSA, 800 mg/l + SNP, 1490 mg/l COC 0.25%	47.0 B	81.3 B

n = 4 plants. Mean separation by Duncan's New Multiple Range Test ($\alpha = 0.05$). Means followed by the same letter are not statistically different.

In the experiment, neither sodium salicylate (NaSA) nor NO alone or in combination with NaSA, caused significant herbicidal damage (lines 1-4). Interestingly, only NaSA significantly potentiated atrazine herbicidal activity on tobacco when used separately (lines 5-7). However, the combination of NO and NaSA decreased salicylate potentiation of atrazine at both 48 and 72 hours post spraying (lines 5 and 8). These results suggest that salicylate and NO are antagonistic. This is similar to what is known from animal systems, where salicylates have been reported to be efficient scavengers of NO in mammalian cells (Hermann *et al*, 1999. FEBS Let. **445**:212), and inhibitors of the transcription of NOS2 (Farivar et al., 1996. JBC **271**:31585).

In plants, the relationship between NO and salicylate is less well understood. However, NO appears to function upstream of salicylate in plant defense, and may inhibit both

catalase and ascorbate peroxidase (Clark et al., 2000. MPMI **13**:1380), which may regulate cellular redox state. Interestingly, SA has been postulated to also inhibit these enzymes as part of its function (Durner *et al.*, 1995. PNAS USA **92**:11312; Durner *et al.*, 1996. JBC **271**:28492). Thus, NO and SA may compete for some molecular targets.

Klepper discusses the implications of his results in the last paragraph of the discussion:

> While salicylic acid has the ability to act as a synergist (for NOx production), this study does not necessarily suggest that salicylic acid can be used successfully in field experiments. Free salicylic acid appears to be short lived in leaf tissue.(footnotes omitted).

This statement suggests that salicylic acid, or by extension its sodium salt (salicylate), would not make good synergists during *in vivo* treatment of plants. However, our results demonstrate that to the contrary, sodium salicylate is an excellent atrazine synergist.

In summary, PSII potentiation by salicylate is not suggested by Klepper. NO_x accumulation is not induced in light by either PSII inhibitors or sodium salicylate. The inhibition of salicylate potentiation of atrazine by NO indicates a separate and distinct mechanism for salicylate activation of herbicidal activity.

7. Although Ryals *et al* demonstrate that SA, acibenzolar, and other compounds are known activators of SAR, the experiments performed by me or under my supervision show that potentiation of PSII inhibitors is independent of Systemic Acquired Resistance (SAR).

We observed potentiation of PS II inhibitors in response to salicylate, acibenzolar-S-methyl, and other substituted salicylates. Although the activity of the selected SAR inducers in inducing SAR may correlate with their ability to potentiate atrazine, this relationship is not necessarily causal. To determine whether SAR is necessary for atrazine potentiation by salicylate, we used the *npr1* mutant of Arabidopsis. The *NPR* gene encodes a transcriptional regulator which controls the ability of salicylate and some SAR inducers to induce pathogenesis-related proteins and immunity to disease (Cao *et al*, 1997. Cell **88**:57). In *npr1* plants,

salicylate is unable to induce PR proteins and disease resistance.

Table 2. Effect of sodium salicylate (NaSA) on atrazine herbicidal activity on Nossen or *npr1-5* Arabidopsis

Treatments	Nossen (WT)	*npr1-5*
Phytotoxicity at 2 day after atrazine application: percent leaf area damaged.		
Crop Oil Concentrate, 0.1% (v/v)	0 A	0 A
NaSA, 400 mg/l + COC 0.1%	12.5 B	7.8 A
Atrazine 100 mg/l + COC 0.1%	0 A	0 A
Atrazine 100 mg/l + 400 mg/l NaSA + COC 0.1%	34.8 CD	23.1 B
Phytotoxicity at 5 days after atrazine application: percent leaf area damaged.		
Crop Oil Concentrate, 0.1% (v/v)	0 A	0 A
NaSA, 400 mg/l + COC 0.1%	13.5 B	6.5 A
Atrazine 100 mg/l + COC 0.1%	26.8 C	14 A
Atrazine 100 mg/l + 400 mg/l NaSA + COC 0.1%	58.5 D	51.5 B
Phytotoxicity at 7 days after atrazine application: percent leaf area damaged.		
Crop Oil Concentrate, 0.1% (v/v)	0 A	0 A
NaSA, 400 mg/l + COC 0.1%	10.8 B	9.5 A
Atrazine 100 mg/l + COC 0.1%	41.5 C	39.0 B
Atrazine 100 mg/l + 400 mg/l NaSA + COC 0.1%	81.3 D	74.0 C
Phytotoxicity at 12 days after atrazine application: percent leaf area damaged.		
Crop Oil Concentrate, 0.1% (v/v)	0 A	0 A
NaSA, 400 mg/l + COC 0.1%	9.0 B	7.8 A
Atrazine 100 mg/l + COC 0.1%	85.3 C	93.8 B
Atrazine 100 mg/l + 400 mg/l NaSA + COC 0.1%	98.8 D	96.9 B

n = 4 plants. Mean separation by Duncan's New Multiple Range Test ($\alpha = 0.05$).

Treatment of *npr1* plants with salicylate and atrazine resulted in atrazine potentiation by salicylate which was very similar to the potentiation that was observed when the Nossen wild type was treated with the same combination. This result is shown in Table 2.

These data demonstrate that SAR is not necessary for salicylate potentiation of atrazine. In fact, the results with the *npr* mutant plants demonstrate that these phenomena are separate.

8. In addition to its role in SAR, SA has several other physiological roles in plants. For example, SA has been shown to be the trigger for thermogenesis in some Arum lilies, an inducer of alternative oxidase in non-overtly thermogenic plants, and it may also regulate ion channels (see Raskin, I. 1992. Ann.Rev. Plant Phys. Plant Mol.Biol. **43**: 439). As such, salicylate has many independent roles in plants that may not be linked through the same signal transduction

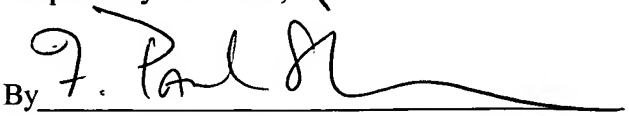
pathways. For example, SA may induce chilling resistance in rice (Kang, et al. 2002. Physiol.Plant. **15**: 571), in a plant where it does not induce SAR (Silverman, et al. (1995) Plant Phys.**108**: 633).

9. To further our understanding of salicylate potentiation of atrazine, we studied the activity of atrazine/salicylate combinations while varying illumination (See Table 7 in the Application) or temperature (See Table 8 in application). In darkness, atrazine was inactive: the leaf damage observed at 12 days was equal to the control. Interestingly, only the combination of atrazine and SA was herbicidal in the dark. Under low light conditions, SA was able to significantly accelerate atrazine herbicidal activity. In contrast, atrazine was only slightly activated at 15° C at low illumination (See Table 8). At either 25° C or 35° C, SA significantly accelerated atrazine activity. SA alone was largely inactive: only at 35° C was significant damage from NaSA sprays observed.

10. In summary, Ryals does not make the invention obvious because SA potentiation of PSII inhibiting herbicides is independent of SAR. Although SA and acibenzolar-S-methyl are both SAR inducers, atrazine potentiation by these compounds is occurring through an SAR-independent pathway. Furthermore, we have observed two unexpected results: 1) a combination of NaSA and atrazine causes plant death in darkness; and 2) under low temperature, atrazine potentiation is abolished. As a PSII inhibiting herbicide, atrazine is only active under illuminated conditions. Extending its activity to kill weed species in darkness may increase its utility as an herbicide.

11. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true, and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that willful false statements may jeopardize the validity of the application or any patent issued thereon.

Respectfully submitted,

By 

F. Paul Silverman

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